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Covalent Modification of the Transactivator Protein IE2-p86 of Human Cytomegalovirus by Conjugation to the Ubiquitin-Homologous Proteins SUMO-1 and hSMT3b

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The 86-kDa IE2 protein (IE2-p86) of human cytomegalovirus (HCMV) is a potent transactivator of viral as well as cellular promoters. Several lines of evidence indicate that this broad transactivation spectrum is mediated by protein-protein interactions. To identify novel cellular binding partners, we performed a yeast two-hybrid screen using a N-terminal deletion mutant of IE2-p86 comprising amino acids 135 to 579 as a bait. Here, we report the isolation of two ubiquitin-homologous proteins, SUMO-1 and hSMT3b, as well as their conjugating activity hUBC9 (human ubiquitin-conjugating enzyme 9) as specific interaction partners of HCMV IE2. The polypeptides SUMO-1 and hSMT3b have previously been shown to be covalently coupled to a subset of nuclear proteins such as the nuclear domain 10 (ND10) proteins PML and Sp100 in a manner analogous to ubiquitinylation, which we call SUMOylation. By Western blot analysis, we were able to show that the IE2-p86 protein can be partially converted to a 105-kDa isoform in a dose-dependent manner after cotransfection of an epitope-tagged SUMO-1. Immunoprecipitation experiments of the conjugated isoforms using denaturing conditions further confirmed the covalent coupling of SUMO-1 or hSMT3b to IE2-p86 both after transient transfection and after lytic infection of human primary fibroblasts. Moreover, we defined two modification sites within IE2, located in an immediate vicinity at amino acid positions 175 and 180, which appear to be used alternatively for coupling. By using a SUMOylation-defective mutant, we showed that the targeting of IE2-p86 to ND10 occurs independent of this modification. However, a strong reduction of IE2-mediated transactivation of two viral early promoters and a heterologous promoter was observed in cotransfection analysis with the SUMOylation-defective mutant. This suggests a functional relevance of covalent modification by ubiquitin-homologous proteins for IE2-mediated transactivation, possibly by providing an additional interaction motif for cellular cofactors.

Human cytomegalovirus (HCMV), a member of the beta subgroup of herpesviruses, is characterized by its narrow host range and prolonged replicative cycle in cell culture as well as in the infected human host. Generally, HCMV possesses low pathogenicity when infecting healthy individuals. However, it is of considerable clinical importance in immunocompromised patients like transplant recipients or patients suffering from AIDS as well as in prenatally infected newborns (2, 3). As found for other herpesviruses, the lytic cycle gene expression of HCMV occurs in a sequential fashion. Initially after infection, the immediate-early (IE) gene products are the first to be synthesized, followed by the early and late gene products (12, 47, 68, 69). IE gene expression, which does not require any prior viral protein synthesis, can be detected from the UL36-38, US3, TRS1, and major IE gene regions (58, 64, 65). The latter encodes two predominant proteins during the IE phase, the 72-kDa IE1 polypeptide (also called IE1-p72 or ppUL123) and the 86-kDa IE2 protein (also called IE2-p86 or ppUL122a) (30, 50, 59). Several additional isoforms of IE2 that arise either via differential splicing or via the usage of a late promoter within the IE2 gene region have been described (50, 52, 59). Both IE1-p72 and IE2-p86 have regulatory functions and have been proposed to play a pivotal role in the discrimination between replication and latency. In particular, IE2-p86 appears to play a master role in triggering the lytic replicative cycle of HCMV (30, 50).

Two main functions of IE2-p86 have been well characterized during the last years. First, this protein is able to repress transcription of its own promoter (29, 51), the potent major IE enhancer-promoter of HCMV (8), thus antagonizing its own expression. This negative autoregulation is mediated by a direct DNA contact of IE2-p86 with a sequence element located between the TATA box and the transcriptional start site of the enhancer-promoter (38, 40). DNA binding of IE2-p86 at this specific position of the promoter has been shown to block the association of RNA polymerase II with the preinitiation complex (39). Second, IE2-p86 is a strong transactivator of viral early promoters and of several heterologous promoters, including the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (26, 35, 43). The transactivating function of IE2-p86 is thought to be required for progression of the replicative cycle from the IE to the early phase. The mechanism of transactivation has not been defined entirely. However, since IE2-p86 interacts with the basal transcription factors TATA-binding protein (26, 56) and TFIIB (10) and with distinct cellular transcription factors such as CREB, AP-1, Egr-1, or Spi-1/PU.1 (37, 55, 67, 74), protein contacts are believed to be essential for transactivation. In addition to the well-characterized functions of IE2 in transactivation and autorepression, the demonstration of interactions with the cell cycle regulatory proteins pRb and p53 suggested that IE2-p86 could also have an influence on cell cycle regulation (24, 57).

Experimental data indicated that IE2-p86 interacts with several additional, as yet unidentified cellular proteins (18).

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Therefore, we decided to perform a yeast two-hybrid screen using a N-terminally truncated IE2-p86 as a bait in order to identify novel binding partners of this important regulatory protein. In this study, we report the isolation of two human ubiquitin-homologous (UbH) polypeptides, SUMO-1 and hSMT3b, and their conjugating activity hUBC9 (human ubiquitin-conjugating enzyme 9) as interaction partners of IE2. We demonstrate that IE2-p86 can be modified by covalent coupling to either SUMO-1 or hSMT3b, resulting in a 105-kDa isoform of IE2 in transient transfection experiments as well as in infected human foreskin fibroblasts (HFFs). Furthermore, we were able to precisely define the modification sites within IE2-p86. Concerning the functional relevance of IE2-p86 conjugation by SUMO-1 or hSMT3b, we demonstrate that modification does not affect the subnuclear distribution of IE2-p86 within nuclear domain 10 (ND10). However, transfection experiments using an IE2 mutant that can no longer by modified by SUMO-1 and hSMT3b suggest that a covalent attachment of UbH proteins to IE2-p86 plays a role for IE2-mediated transactivation.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were obtained from Eurogentec (Seraing, Belgium) and ARK (Darmstadt, Germany). The following oligonucleotides (5′ to 3′ sequences) were used for cloning and in vitro mutagenesis: IE2pACT5 (AGCTGGATCCTGGGGCATCCGCTACTCCCGA), IE2pACT3 (AGTCGGATCCGAGACTTGTTCCTCAGGTCC), pSC5 (ACCCAAGCTTGGTACCGAGCTCCGCACC), pSC3 (CTAGTCTAGACTGACTAACTAGATGCATGC), SUMOflag5 (AGTCGGATCCTCTGACCAGGAGGCAAAACCT), SUMOflag3 (AGTCTCTAGACTAAACTGTTGAATGACCCCC), SMT3flag5 (AGTCGGATCCGCCGAAAAGCCCAAG), SMT3flag3 (AGTCTCTAGATCAGTAGACACCTCCCGTCTG), IE2-N1 (AGCGAAGCTTGTCGACCTCGAGCCACCAT), IE2-N2 (AGCGAAGCTTGTCGACCTGCGGCCATCAGAGC), IE2-N3 (AGCGAAGCTTGTCGACCCGCATCTCCGAGTTG), C-IE2 (AGCTGAATTCAAGCTTCCCCACGGGTAGGC), 5IE2mut175 (ATGCTGCCCCTCATCCGACAGGAAGACATCAAG), 3IE2mut175 (CTTGATGTCTTCCTGTCGGATGAGGGGCAGCAT), 5IE2mut180 (AAACAGGAAGACATCCGACCCGAGCCCGACTTT), 3IE2mut180 (AAAGTCGGGCTCGGGTCGGATGTCTTCCTGTTT), IE2-180mut175-5 (ATGCTGCCCCTCATCCGACAGGAAGACATCCGA), IE2-180mut175-3 (ACGGATGTCTTCCTGTCGGATGAGGGGCAGCAT), IE2-pQE16A (AGTCGGATCCATGGAGTCCTCTGCCAAGAG), IE2fImut3 (AGTCGAATTCGAGGGGCAGCATGATAGG), IE2fIImut5 (AGTCGAATTCGAGCCCGACTTTACCATC), IE2fIImut3 (AGTCTCTAGATTACTGAGACTTGTTCCTCAG), PR-ul84A (GATCGCTAGCGGTGGCCAACCTAGCCTCGG), and PR-ul84B (CAGCCTCGAGTTGGTGTCTGCGGGCGCGAG).

Plasmid constructions and in vitro mutagenesis. The bait plasmid pHM247 for the yeast two-hybrid screen was constructed by isolation of a *Sma*I/*Sal*I fragment from plasmid pHM134 containing the IE2-p86 cDNA (37) followed by Klenow treatment and insertion into the *Nco*I restriction site of the GAL4 DNA-binding domain vector pAS1 (15), which had also been filled in by treatment with Klenow enzyme. The yeast vector pHM704 expressing the same IE2-p86 sequence as fusion with the GAL4 activation domain was constructed by PCR amplification using oligonucleotides IE2pACT5 and IE2pACT3, followed by cleavage with *Bam*HI and ligation with the pACT vector (15). The resulting GAL4 fusion proteins comprise amino acids 135 to 579 of IE2-p86. An expression vector for a GAL4 activation domain fusion protein containing the viral open reading frame UL84 was created by cleavage of vector pcDNAUL84 (19) with *Xba*I followed by Klenow treatment and thereafter release of the UL84 fragment by cleavage with *Eco*RI. The GAL4 activation domain vector pGAD424 (Clontech, Palo Alto, Calif.) was prepared by cleavage with *Sal*I followed by Klenow treatment and restriction with *Eco*RI. Vector and insert were then ligated, resulting in plasmid pHM379.

To construct a eucaryotic expression vector for epitope-tagged proteins, the coding sequence for the FLAG epitope was amplified by PCR using primers pSC5 and pSC3 and plasmid pSuperCATCH (20) as the template. Conditions for PCR were as described previously (19). The amplified fragment was cleaved with *Hin*dIII/*Xba*I followed by insertion into the pcDNA3 vector (Invitrogen Corp., San Diego, Calif.), resulting in plasmid FLAG-pcDNA3. The SUMO-1 and hSMT3b cDNAs were amplified by PCR from one of the library clones obtained in the yeast two-hybrid screen, using either oligonucleotides SUMOflag5 and SUMOflag3 or oligonucleotides SMT3flag5 and SMT3flag3 as primers. The resulting fragments were inserted into the FLAG-pcDNA3 vector via *Bam*HI/*Xba*I sites.

For mapping of sites modified by SUMO-1 (i.e., SUMOylation sites), the IE2 deletion mutants IE2(290-548), IE2(310-548), and IE2(329-548) were generated by PCR amplification using plasmid pHM134 as a template and oligonucleotides C-IE2 plus IE2-N1, IE2-N2, and IE2-N3, respectively. The PCR products were cleaved with *Sal*I and *Eco*RI and ligated with the pSuperCATCH-NLS vector (20). Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit as instructed by the manufacturer (Stratagene, Heidelberg, Germany). The IE2-p86 single amino acid mutants were constructed using oligonucleotides 5IE2mut175 and 3IE2mut175 (resulting in the mutant IE2mut175 carrying a K-R substitution at amino acid 175 of IE2-p86) or 5IE2mut180 and 3IE2mut180 (resulting in the mutant IE2mut180 with a K-R substitution at amino acid 180 in IE2-p86). The IE2 double amino acid mutant IE2mut175+180 was created by using the IE2mut180 plasmid as the template and oligonucleotides IE2-180mut175-5 and IE2-180mut175-3. The internal IE2 deletion mutant IE2del174-181 was constructed by PCR amplification of an N-terminal IE2 fragment (nucleotides 139 to 658) using the oligonucleotides IE2-pQE16A and IE2fImut3 and a C-terminal IE2 fragment (nucleotides 679 to 1953) using the oligonucleotides IE2fIImut5 and IE2fIImut3. Thereafter, the N-terminal fragment was inserted into the pcDNA3 vector using the *Bam*HI and *Eco*RI restriction sites, followed by ligation with the C-terminal fragment via *Eco*RI/*Xba*I, thus resulting in an internal deletion of amino acids 174 to 181 in IE2-p86. The internal IE2 deletion mutant IE86ΔSX was obtained from D. Spector (San Diego, Calif.) (56). Construction of a vector expressing IE2 in fusion with the green fluorescent protein (GFP) will be described elsewhere. The IE1 expression vector pHM494 was created by isolation of the IE1 cDNA from plasmid pHM124 followed by insertion into the pcDNA3 vector via *Hin*dIII/*Eco*RI (52). The luciferase expression plasmids pHM142 containing the UL112/113 promoter and pHIV-luc containing the HIV LTR have been described previously (5, 19). The luciferase expression plasmid pHM571 containing the UL84 promoter was constructed by PCR amplification of the UL84 promoter sequence (nucleotides 123072 to 123404) using oligonucleotides PR-ul84A and PR-ul84B and cosmid pCM1106 (17) as the template followed by ligation with luciferase expression vector pGL3basic (Promega, Mannheim, Germany) via *Nhe*I/*Xho*I. The DNA sequence of each plasmid construct was confirmed by automated sequence analysis (ABI, Weiterstadt, Germany).

Yeast two-hybrid screening. Yeast two-hybrid screening was performed using GAL4 fusion proteins as described previously (16). *Saccharomyces cerevisiae* Y153 was transformed by the lithium acetate method using the bait plasmid pHM247 (21). The presence of pHM247 in the yeast cells was stably maintained by selection for tryptophan prototrophy. Expression of the GAL4-IE2 fusion protein was confirmed by Western blot analysis using the IE2-p86 antiserum. Y153 yeast cells transfected with pHM247 alone or in combination with pACT were then tested for activation of the reporter genes *HIS3* and *lacZ*. In neither case was activation of the reporter genes by GAL4-IE2 observed. Yeast strain Y153 containing pHM247 was subsequently transformed with a cDNA library derived from human B lymphocytes fused to the GAL4 activation domain in the pACT vector (15). Primary transformants (10^7) were selected for growth on histidine dropout plates containing 25 mM 3-aminotriazole. His^+ colonies were subsequently analyzed for β-galactosidase activity by filter test experiments (9). The interaction was then quantified by *o*-nitrophenyl galactopyranoside (ONPG) assays as described elsewhere (23). Interactor plasmids from clones positive in both tests were rescued by transformation of competent *Escherichia coli* KC8 with total yeast DNA (31). Interactor plasmids derived from double-positive clones were tested for bait specificity by retransformation with the GAL4 plasmid pAS1. The nucleotide sequences of the cDNA inserts were determined by automated sequence analysis (ABI).

Cell culture, transfection, and reporter assays. HFFs were cultured as described previously (5). U373MG and 293 cells were obtained from the American Type Culture Collection (Manassas, Va.) and maintained in Dulbecco's minimal essential medium (Gibco/BRL, Eggenstein, Germany) supplemented with 8 and 10% fetal calf serum, respectively. The day before transfection, HFFs were plated onto 100-mm-diameter plastic dishes at 1.3×10^6 cells per dish. 293 cells were seeded onto six-well plates at 3×10^5 cells per well 2 days before transfection. Plasmid transfections were performed by the calcium phosphate coprecipitation procedure using *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid as described elsewhere (19) and a total of 10 μg of DNA for 100-mm-diameter dishes or 1 to 2 μg of DNA for six-well plates. Cells were harvested 48 h after transfection and used for Western blotting or immunoprecipitation. For indirect immunofluorescence analysis, HFFs grown on coverslips were transfected with 2 μg of plasmid DNA using the FuGENE transfection reagent as specified by the manufacturer (Boehringer, Mannheim, Germany).

For luciferase assays, U373MG cells were plated onto six-well dishes at 2.8×10^5 cells per well the day before transfection. Plasmid transfection was performed by the DEAE-dextran method as described previously (5). Routinely, 1 μg of luciferase target and 2.3 μg of the cotransfected transactivator plasmid were used. The total amount of transfected DNA was kept constant by using the cloning vector pCB6 in order to replace the missing transactivator plasmid. At 48 h after transfection, cells were harvested and luciferase assays were performed using a lysis buffer containing 50 mM Tris-H_3PO_4 (pH 7.8), 50 mM trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2% Triton X-100, 4 mM dithiothreitol, and 20% glycerol. Luciferase activity in the supernatant was determined using a luminometer (Bertholt, Freiburg, Germany). Each transfection was performed in triplicate and was repeated at least three times.

Antibodies. The polyclonal antisera against IE2-p86 (19), exon 5 of IE2-p86 (referred to as anti-pHM178), or pUL84 of HCMV were generated by immunizing rabbits with the respective procaryotically expressed proteins. Monoclonal antibody (MAb) 810, which recognizes an epitope contained within exon 2 of both IE1-p72 and IE2-p86 (46), was obtained from Chemicon (Hofheim, Germany). The anti-FLAG MAb M2, which is directed against the synthetic FLAG octapeptide N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C, was purchased from INTEGRA Bioscience (Fernwald, Germany). MAb p63-27 directed against IE1-p72 and the anti-Sp26 antiserum directed against Sp100 (kindly provided by T. Sternsdorf, Hamburg, Germany) were described previously (4, 61). MAb 21C7 directed against SUMO-1 (45) was purchased from Zymed Laboratories (Labor Diagnostika, Heiden, Germany). Anti-mouse and anti-rabbit horseradish peroxidase- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were obtained from Dianova (Hamburg, Germany).

Western blotting and immunoprecipitation analysis. Initially, transfected cells were lysed in NP-40 lysis buffer as described previously (6). For inhibition of deSUMOylation, transfected or infected cells were lysed in 120 μl of a 1:3 dilution of buffer I (5% sodium dodecyl sulfate [SDS], 0.15 M Tris [pH 6.7], 30% glycerol) and buffer II (25 mM Tris [pH 8.2], 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS, 0.1% azide, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg each of aprotinin, leupeptin, and pepstatin per ml) essentially as described elsewhere (13). After a brief sonification, lysates were diluted in SDS Laemmli buffer and boiled at 94°C for 10 min. Samples were subjected to polyacrylamide gel electrophoresis (PAGE) on SDS–8 to 10% polyacrylamide gels, and the proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Western blotting and enhanced chemiluminescence (ECL) detection were performed according to the manufacturer's protocol (ECL Western detection kit; Amersham Pharmacia Biotech Europe, Freiburg, Germany). Coimmunoprecipitation analysis for detection of noncovalent protein interactions was performed as described elsewhere (6). For detection of covalent modifications by SUMO-1 or hSMT3b, total cell lysates were prepared essentially as described elsewhere (13). Transfected or infected cells were lysed as described above. After a brief sonification, the lysates were diluted 1:10 in phosphate-buffered saline (PBS)–0.5% NP-40 followed by high-speed centrifugation at room temperature. The supernatant was incubated with the appropriate antibody for 1 h at room temperature; then a 50% protein A-Sepharose suspension was added for another 2 h at room temperature. The Sepharose beads were collected and washed three times in PBS–0.5% NP-40. Antigen-antibody complexes were recovered by boiling in SDS sample buffer and analyzed by Western blotting.

Indirect immunofluorescence analysis. For indirect immunofluorescence analysis, HFF cells on coverslips were washed two times with PBS followed by fixation with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized in PBS–0.2% Triton X-100 on ice for 20 min. Thereafter, the cells were incubated for 30 min at 37°C with a 1:2,500 dilution of MAb 810 (directed against IE1-p72 and IE2-p86) and a 1:200 dilution of anti-Sp26 (directed against Sp100) in PBS, followed by incubation with anti-mouse FITC and anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies. Cells were mounted using Vectashield mounting medium including 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif.) and analyzed using a Zeiss Axiovert-135 microscope. Images were recorded with a cooled MicroMax charge-coupled device camera (Princeton Instruments, Trenton, N.J.) and processed by using IPLab Spectrum and Adobe Photoshop package (Scanalytics Inc., Fairfax, Va.; Adobe Systems Incorporated).

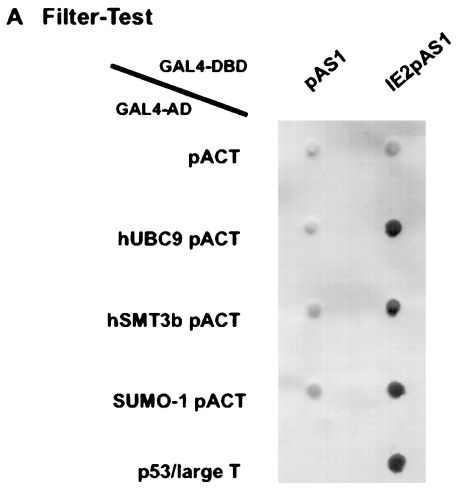
RESULTS

Identification of SUMO-1, hSMT3b, and hUBC9 as cellular interaction partners of the HCMV IE2 protein by yeast two-hybrid experiments. To identify novel cellular interaction partners of the IE2 protein of HCMV, a yeast two-hybrid screen was carried out. Since the full-length IE2-p86 activated the reporter genes *HIS3* and *lacZ* in yeast by itself, an N-terminal deletion mutant of IE2-p86 comprising amino acids 135 to 579 was used as a bait in fusion with the DNA-binding domain of the yeast factor GAL4 in the pAS1 vector (pHM247). The presence of the GAL4-IE2 expression plasmid pHM247 was maintained stably in yeast strain Y153 by selection in liquid dropout culture medium lacking tryptophan, and expression of the relevant fusion protein was confirmed by Western blot analysis (data not shown). To determine whether the bait protein was able to interact with already known binding partners in yeast, the pHM247-containing yeast strain Y153 was transformed with plasmid pHM379 or pHM704, containing either the UL84 protein of HCMV or an N-terminally truncated IE2 protein (amino acids 135 to 579) in fusion with the GAL4 activation domain, respectively. In either case, the reporter genes *HIS3* and *lacZ* were activated, indicating that the IE2 deletion mutant was able to interact with pUL84 and was also able to dimerize as reported in previous publications (data not shown) (11, 19).

The yeast two-hybrid screen was performed by transformation of yeast strain Y153 containing plasmid pHM247 with a cDNA library derived from B lymphocytes in the vector pACT (15). This particular library was chosen since it is highly complex and has previously been used by several groups for the successful isolation of interacting proteins (15). Moreover, since IE2-p86 is able to transactivate in a broad spectrum of different cell types, our main interest was to select ubiquitously expressed proteins with a role in IE2 protein function. By using this screening procedure, 1.25×10^7 primary transformants were tested for interaction with IE2 by selection for histidine prototrophy on dropout plates supplemented with 25 mM 3-aminotriazole and by expression of β-galactosidase as determined by filter lift assays. Plasmids encoding putative interactors of IE2 were isolated from double-positive clones and retransformed into yeast strain Y153/pHM247 in order to confirm the interaction. Positive clones after this retransformation were characterized by automated sequencing and search for homologies in the National Center for Biotechnology Information databases. In total, we were able to identify 15 cellular proteins that showed an interaction with IE2 in yeast. Here, we report the identification of SUMO-1, hSMT3b, and hUBC9 as specific interaction partners of the IE2 protein (Fig. 1). For these interaction partners, more than one copy was found in the yeast two-hybrid screen, indicating a sufficient complexity of the cDNA library and the specificity of the interaction with IE2. By cotransformation experiments of the individual interactor clones and the empty pAS1 vector, it was excluded that SUMO-1, hSMT3b, and hUBC9 were able to activate the reporter genes in yeast in the absence of a bait protein (Fig. 1A). Additionally, liquid β-galactosidase assays (ONPG assays) were performed to quantify the strength of interaction with IE2 (Fig. 1B). Interestingly, the interaction between IE2 and SUMO-1 turned out to be even stronger than the interaction between p53 and the simian virus 40 (SV40) T antigen, which served as a positive control.

The interactors SUMO-1 and hSMT3b belong to the family of UbH proteins (7, 44). They were shown to be covalently attached to a variety of nuclear targets in a manner analogous to protein ubiquitinylation (32, 34), which involves hUBC9 (32, 54). Thus, the simultaneous identification of SUMO-1, hSMT3b, and hUBC9 as interacting proteins of IE2 suggested that IE2-p86 might be a target for posttranslational modification by coupling to SUMO-1 or hSMT3b.

Evidence for a covalent modification of IE2 by SUMO-1 and hSMT3b. Having identified the ubiquitin-like proteins SUMO-1 and hSMT3b as potential interaction partners of HCMV IE2 in the yeast two-hybrid screen, we searched for physiological situations in mammalian cells in which IE2-p86 is covalently modified by SUMO-1 or hSMT3b. For this purpose, both the SUMO-1 and the hSMT3b cDNA were expressed as FLAG-tagged proteins using the FLAG-pcDNA3 expression vector. 293 cells were transfected with the FLAG-hSMT3b or, as shown in Fig. 2, with the FLAG-SUMO-1 expression vector either alone or in combination with the IE2-p86 expression plasmid pHM134. After lysis of cells using NP-40 buffer, proteins were analyzed by Western blotting. The IE2 protein was detected using the anti-IE86 rabbit serum (19) (Fig. 2A). In lysates from cells expressing IE2-p86 and increasing amounts of FLAG-SUMO-1, the IE2 86-kDa protein was detected as well as an additional 105-kDa isoform which is fairly consistent with a 20-kDa covalent modification of IE2-p86 by FLAG-



B ONPG-Assay

pAS1	IE2 pAS1
0.4	0.2
0.4	40.1+/- 4.58
0.4	2.8 +/- 0.57
0.5	165.5 +/- 45.8
	100

FIG. 1. Specific interaction between HCMV IE2(135-579) and hUBC9, hSMT3b, and SUMO-1 in yeast. Yeast cells were transformed with two separate vectors, one of which encoded either IE2(135-579) fused to the GAL4 DNA-binding domain (IE2pAS1) or the DNA-binding domain alone (pAS1). The second plasmid encoded either the GAL4 activation domain alone (pACT) or hUBC9, hSMT3b, and SUMO-1 as fusions with the GAL4 activation domain, (plasmids hUBC9 pACT, hSMT3b pACT, and SUMO-1 pACT, respectively). Yeast colonies were selected for the presence of both plasmids with dropout media lacking tryptophan and leucine and subsequently analyzed for the expression of β-galactosidase. The association of murine p53 (encoded by plasmid pVA3; Clontech) and SV40 large T antigen (plasmid pTD1; Clontech) served as a positive control. (A) Qualitative analysis of interactions between IE2 (135-579) and hUBC9, hSMT3b, and SUMO-1 as determined in filter lift experiments after staining for β-galactosidase activity. (B) Quantitation of the association between IE2(135-579) and hUBC9, hSMT3b, and SUMO-1 as determined by liquid β-galactosidase assays (ONPG assays). Cotransformation experiments were performed as for panel A. β-Galactosidase activity was assayed from liquid cultures in at least three independent experiments, each with duplicate samples. The β-galactosidase activity of yeast cells transformed with a vector combination encoding murine p53 and SV40 large T antigen was set as 100.

SUMO-1. After removal of the IE2-specific antibodies, the same membrane was incubated with MAb M2 directed against the FLAG epitope in order to investigate whether this 105-kDa band contained FLAG-SUMO-1 (Fig. 2B). In lanes 2 and 4 to 6 we observed a smear toward higher-molecular-weight proteins representing FLAG-SUMO-1-conjugated cellular proteins as described previously (42, 45). The 90-kDa band which could be detected in the absence as well as in the presence of cotransfected IE2 most probably represents the p90 form of RanGAP1 (45). Only in the presence of IE2-p86 did we detect an additional immunoreactive band of 105 kDa which perfectly overlapped with the IE2-immunoreactive 105-kDa band shown in Fig. 2A.

Referring to experiments as described by Desterro et al. (13), in which IκBα–SUMO-1 conjugates were rarely detectable after cell lysis using NP-40 lysis buffer, we performed

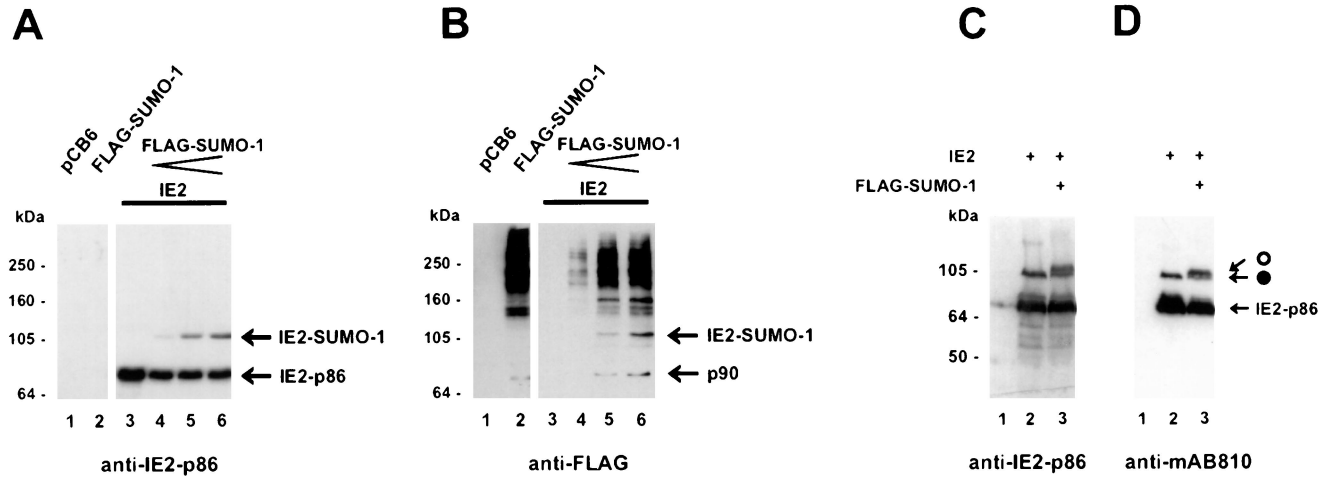


FIG. 2. Covalent interaction between IE2-p86 and FLAG-SUMO-1 in 293 cells. 293 cells were transfected either with the empty eucaryotic expression vector pCB6, the FLAG-SUMO-1 expression vector, or plasmid pHM134 (encoding IE2-p86) either alone or in combination as indicated. Cells in panels A and B were lysed using NP-40 lysis buffer, whereas lysates in panels C and D were prepared under denaturing conditions using SDS lysis buffer. The lysates were fractionated by SDS-PAGE (8% gel) and analyzed by Western blotting. (A) Western blot analysis of cell lysates using the IE2-p86 polyclonal antiserum. (B) Incubation of the same filter membrane as in panel A, using the anti-FLAG MAb. Lanes: 1, transfection with expression vector pCB6 alone; 2, transfection with the FLAG-SUMO-1 expression vector alone; 3, transfection with plasmid pHM134 (encoding IE2-p86) alone; 4 to 6, transfection with a constant amount of plasmid pHM134 and increasing amounts of plasmid pFLAG-SUMO-1. (C and D) Western blot analyses of cell lysates using the IE2-p86 polyclonal antiserum (C) or MAb 810 (D). Lanes: 1, transfection with vector pCB6 alone; 2, transfection with plasmid pHM134 alone; 3, transfection with a combination of plasmids pHM134 and pFLAG-SUMO-1. The IE2-p86 modification by an endogenous UbH moiety is indicated by a closed circle; the modification by cotransfected FLAG-SUMO-1 is depicted by an open circle.

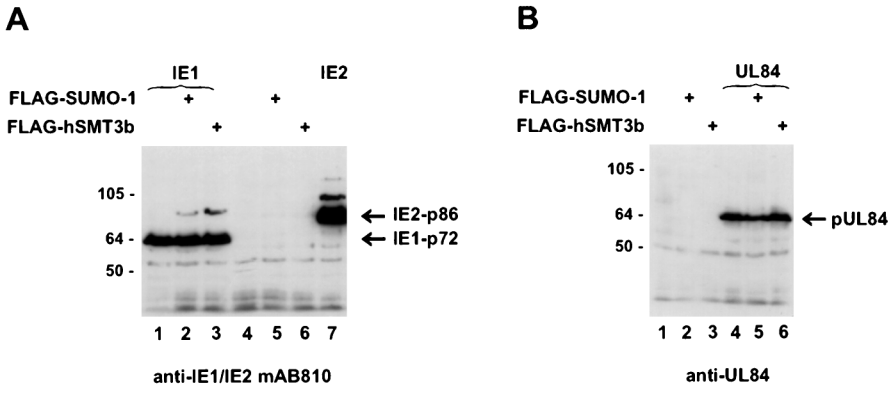


FIG. 3. Analysis of covalent modification of HCMV IE1-p72 and pUL84 by FLAG-SUMO-1 and FLAG-hSMT3b, respectively. 293 cells were transfected with expression vectors encoding IE1-p72, pUL84, or FLAG-SUMO-1/hSMT3b as indicated and lysed in SDS lysis buffer. Cell extracts were resolved by SDS-PAGE (10% gel) and subjected to immunoblotting using MAb 810 directed against a N-terminal epitope shared by IE1-p72 and IE2-p86 (A) or a polyclonal antiserum directed against HCMV pUL84 (B). (A) Lanes: 1, transfection with the IE1-p72 expression vector alone; 2, transfection with a combination of vectors encoding IE1-p72 and FLAG-SUMO-1; 3, transfection with a combination of vectors encoding IE1-p72 and FLAG-hSMT3b; 4, transfection with vector pCB6 alone; 5, transfection with plasmid pFLAG-SUMO-1 alone; 6, transfection with plasmid pFLAG-hSMT3b alone; 7, transfection with plasmid pHM134 (encoding IE2-p86) alone. (B) Lanes: 1, transfection with vector pCB6 alone; 2, transfection with plasmid pFLAG-SUMO-1 alone; 3, transfection with plasmid pFLAG-hSMT3b alone; 4, transfection with UL84 expression vector pcDNAUL84 alone; 5, transfection with a combination of pcDNAUL84 and pFLAG-SUMO-1; 6, transfection with a combination of pcDNAUL84 and pFLAG-hSMT3b. Sizes are indicated in kilodaltons.

analogous transfection experiments but lysed the cells in 1.7% SDS buffer (13). Again, Western blot analyses were performed with the IE2-p86 antiserum (Fig. 2C). Under conditions of SDS lysis, a 105-kDa IE2 isoform was already observable in the absence of FLAG-SUMO-1, whereas after cotransfection with FLAG-SUMO-1 we detected a second band with slightly slower electrophoretic mobility, representing the IE2/FLAG-SUMO-1 conjugate because of additional amino acids derived from vector sequences and the FLAG epitope (Fig. 2C and D, lanes 2). To exclude artifacts from the antiserum, we repeated this experiment using the same lysates and MAb 810, which is directed against an amino-terminal epitope contained within both IE1-p72 and IE2-p86 (45) (Fig. 2D). Similarly, an endogenous modification of IE2 was visible after transfection of the IE2-p86 expression vector pHM134 alone, which resolved in a double band after additional cotransfection of FLAG-SUMO-1. Analogous cotransfection experiments with IE2-p86 and FLAG-hSMT3b followed by Western blot analysis showed comparable results (data not shown). Taken together, these experiments indicate that IE2-p86 can be covalently modified after coexpression of FLAG-SUMO-1 or FLAG-hSMT3b. Furthermore, an IE2 isoform of similar molecular weight can be observed when IE2 is expressed alone in 293 cells.

As the major IE proteins IE1-p72 and IE2-p86 of HCMV have the first 85 amino acids in common, we wished to determine whether IE1-p72 is also modified by SUMO-1 or hSMT3b. Therefore, analogous cotransfection experiments were performed with the IE1-p72 expression vector pHM494 and FLAG-SUMO-1 or FLAG-hSMT3b in 293 cells. Both HCMV IE proteins were detected using MAb 810. As is evident from Fig. 3A, lanes 2 and 3, IE1-p72 could likewise be covalently modified by both FLAG-SUMO-1 and FLAG-hSMT3b. However, in contrast to IE2-p86 (Fig. 3A, lane 7), no endogenous modification of IE1-p72 was detectable in the absence of the FLAG-UbH proteins under these conditions.

This result led us to question whether SUMOylation after overexpression is a general feature of viral regulatory proteins. Therefore, we tested if modification of three additional viral proteins could be observed in the presence of FLAG-SUMO-1 or FLAG-hSMT3b. However, in contrast to IE1-p72 and IE2-p86, no conjugation was detectable for the viral protein pUL26, pUL69 (data not shown), or pUL84 (Fig. 3B), indicating that SUMOylation is restricted to the major IE proteins of HCMV.

Immunoprecipitation of IE2-UbH conjugates under denaturing conditions confirms the covalent nature of the interaction. To further strengthen the evidence for a covalent modification of IE2-p86 by SUMO-1 and hSMT3b, we performed cotransfection experiments in 293 cells followed by immunoprecipitation with the IE2-p86 antiserum. Conjugates between IE2-p86 and either FLAG-SUMO-1 or hSMT3b were then detected by Western blot analyses of the precipitates using the anti-FLAG MAb (Fig. 4). Here as well as in the following immunoprecipitation experiments, an aliquot of each sample was analyzed by Western blotting prior to immunoprecipitation in order to confirm that equal amounts of protein had been expressed (data not shown). The well-characterized interaction between IE2 and the UL84-protein of HCMV (19) served as a positive control (Fig. 4A and B, lanes 1). As the binding of IE2-p86 to pUL84 is of noncovalent nature, cells were lysed using NP-40 lysis buffer, whereas cells expressing IE2 and FLAG-SUMO-1 or FLAG-hSMT3b were lysed essentially as described by Desterro et al. (13). As shown in Fig. 4A, lane 2, the noncovalent interaction between IE2 and pUL84 was not observed under those denaturing conditions. On the other hand, the attachment of FLAG-hSMT3b to IE2 was detected after cotransfection of a constant amount of the IE2-p86 expression vector in combination with increasing amounts of the FLAG-hSMT3b construct (Fig. 4A, lanes 5 to 7). Surprisingly, two additional bands with calculated molecular masses of about 130 and 150 kDa could be observed apart from the 105-kDa IE2-hSMT3b conjugate in Western blot analysis (Fig. 4A, lanes 5 to 7).

Analogous experiments were performed after cotransfection of IE2-p86 and FLAG-SUMO-1 (Fig. 4B). Again, the 105-kDa IE2-SUMO-1 conjugate was detected by the anti-FLAG antibody in Western blot analysis (Fig. 4B, lane 3); two additional bands with calculated molecular masses of 130 and 150 kDa were also present (Fig. 4B, lane 3, indicated by asterisks). These two bands seem to be specific because they were observed only in the presence of both IE2-p86 and FLAG-SUMO-1 or FLAG-hSMT3b and were not observed in cell

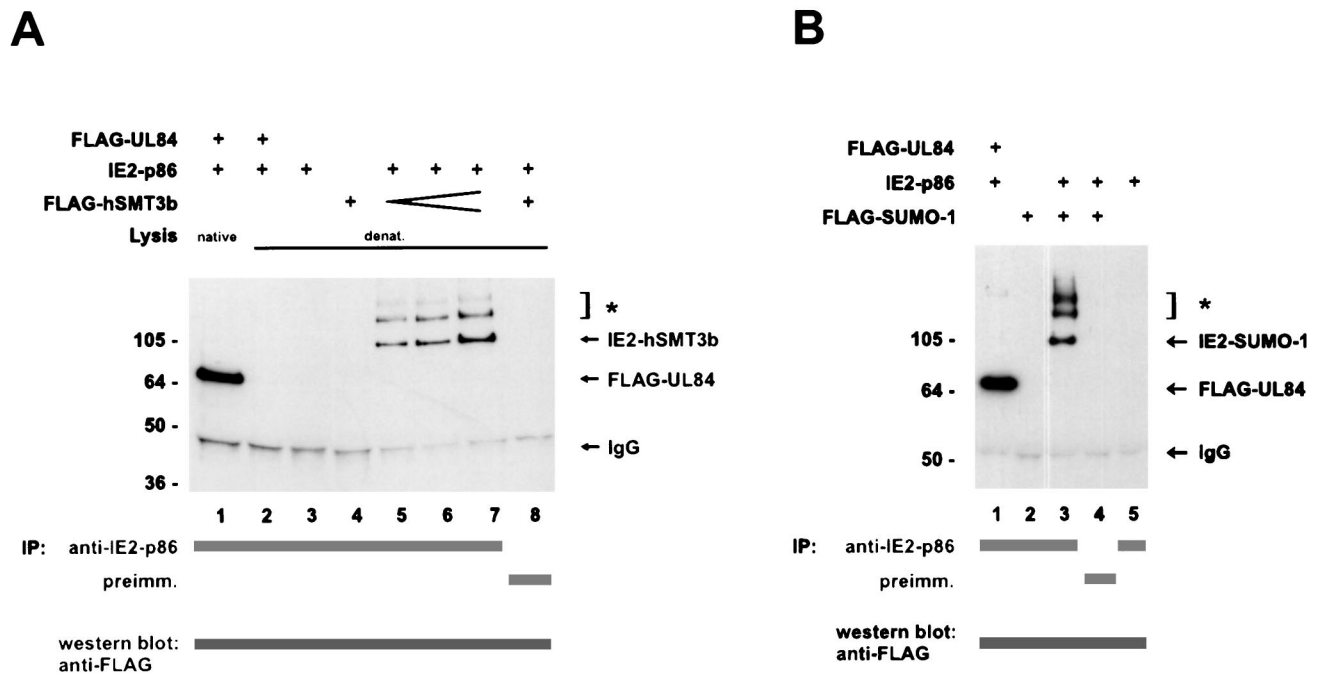


FIG. 4. Evidence for a covalent modification of HCMV IE2-p86 by SUMO-1 and hSMT3b in immunoprecipitation experiments. 293 cells were transfected with an expression vector encoding IE2-p86, FLAG-SUMO-1/hSMT3b, or FLAG-UL84 as indicated and prepared for immunoprecipitation as described in Materials and Methods. Immunoprecipitations were performed with the IE2-p86 antiserum or the IE2-p86 preimmune serum, as indicated by bars. Precipitates were washed three times and separated by SDS-PAGE (10% gel). Thereafter, coprecipitated interactor proteins were detected by Western blot analysis using the anti-FLAG MAb. The interaction between IE2-p86 and FLAG-UL84 served as a positive control. Asterisks depict the two additional anti-FLAG reactive bands in IE2-UbH precipitates. (A) Western blot analysis of precipitated IE2-hSMT3b conjugates. Lanes: 1, cell lysis under native lysis conditions; 2 to 8, cell lysis under denaturating conditions. Transfection was performed with plasmids encoding FLAG-UL84 and IE2-p86 (lanes 1 and 2), IE2-p86 alone (lane 3), FLAG-hSMT3b alone (lane 4), and IE2-p86 and FLAG-hSMT3b (lanes 5 to 8). (B) Western blot analysis of precipitated IE2-SUMO-1 conjugates. Lanes: 1, cell lysis under native lysis conditions; 2 to 5, cell lysis under denaturating conditions. Transfection was performed with plasmids encoding FLAG-UL84 and IE2-p86 (lane 1), FLAG-SUMO-1 alone (lane 2), FLAG-SUMO-1 and IE2-p86 (lanes 3 and 4), and IE2-p86 alone (lane 5). Here and in subsequent figures, sizes are indicated in kilodaltons, and IgG and IP stand for immunoglobulin G and immunoprecipitation, respectively.

lysates either expressing IE2-p86 or the FLAG-UbH protein alone. Surprisingly, however, these bands could not be detected when Western blots were performed with IE2-specific MAbs or sera, suggesting that they do not represent additional IE2 conjugates (data not shown; Fig. 5C). In summary, these results confirm a covalent conjugation of FLAG-SUMO-1 and FLAG-hSMT3b to the HCMV IE2-p86 protein.

Covalent modification of IE2-p86 by SUMO-1/hSMT3b occurs also during the replicative cycle of HCMV. Having demonstrated that HCMV IE2-p86 is conjugated by UbH proteins in 293 cells, we wanted to investigate whether a similar modification also occurs in infected primary HFFs. Therefore, we analyzed lysates from infected HFFs 6, 20, 27, 48, and 72 h after infection by Western blotting using the anti-pHM178 rabbit antiserum which is directed against amino acid sequences encoded by exon 5 of IE2-p86. As shown in Fig. 5A, a 105-kDa IE2 subform was also observed during the whole time course of lytic infection, detectable already at 6 h after infection. This subform is similar in electrophoretic mobility to extracts from IE2-expressing 293 cells (Fig. 5A, lanes 8 and 9).

To further determine whether SUMO-1 or hSMT3b modification of IE2-p86 occurs in infected cells, HFFs were transfected with FLAG-SUMO-1 or FLAG-hSMT3b. The day after transfection, cells were either mock infected or infected with HCMV for 72 h. Thereafter, immunoprecipitations were performed with MAb 810. In Western blot analysis with the anti-FLAG MAb, a 105-kDa FLAG-reactive band was detected in precipitates containing either FLAG-SUMO-1 or FLAG-hSMT3b; as already observed after immunoprecipitation analysis of 293 cell lysates, the two higher-molecular-mass forms of 130 and 150 kDa could also be detected (Fig. 5B, lanes 2 and 3). To determine which bands as detected by the anti-FLAG antibody contained the IE2 protein, an identical immunoprecipitation with MAb 810 was performed followed by Western blotting using the IE2-p86 specific rabbit antiserum. As can be seen in Fig. 5C, lanes 2 and 3, the 86- and 105-kDa IE2 isoform, but no higher-molecular-mass bands of 130 or 150 kDa, were detected with the IE2-specific antiserum, indicating that the higher-molecular-mass anti-FLAG reactive bands do not represent additional IE2 isoforms. In contrast, no modification of IE1-p72 could be observed during viral infection using the same immunoprecipitation conditions followed by Western blot analysis with an IE1-specific MAb (Fig. 5D, lanes 2 and 3). These experiments confirm that IE2-p86 modification by SUMO-1 or hSMT3b can also occur in infected human fibroblasts. Furthermore, the modification of the two major IE proteins of HCMV seems to be differentially regulated, as IE2-p86 is modified during the complete replication cycle whereas IE1-p72 conjugates are not detectable at 72 h after infection under those conditions.

Mapping of the SUMO-1/hSMT3b conjugation site within the HCMV IE2 protein identifies lysine residues 175 and 180 as two independent acceptor sites for modification. Having shown that IE2-p86 can be modified by both SUMO-1 and hSMT3b in 293 cells as well as in infected HFFs, we were interested in mapping the precise conjugation site within IE2-p86. For this, a series of N- and C-terminal IE2 deletion mutants was created (Fig. 6A). The C-terminal deletion mutants

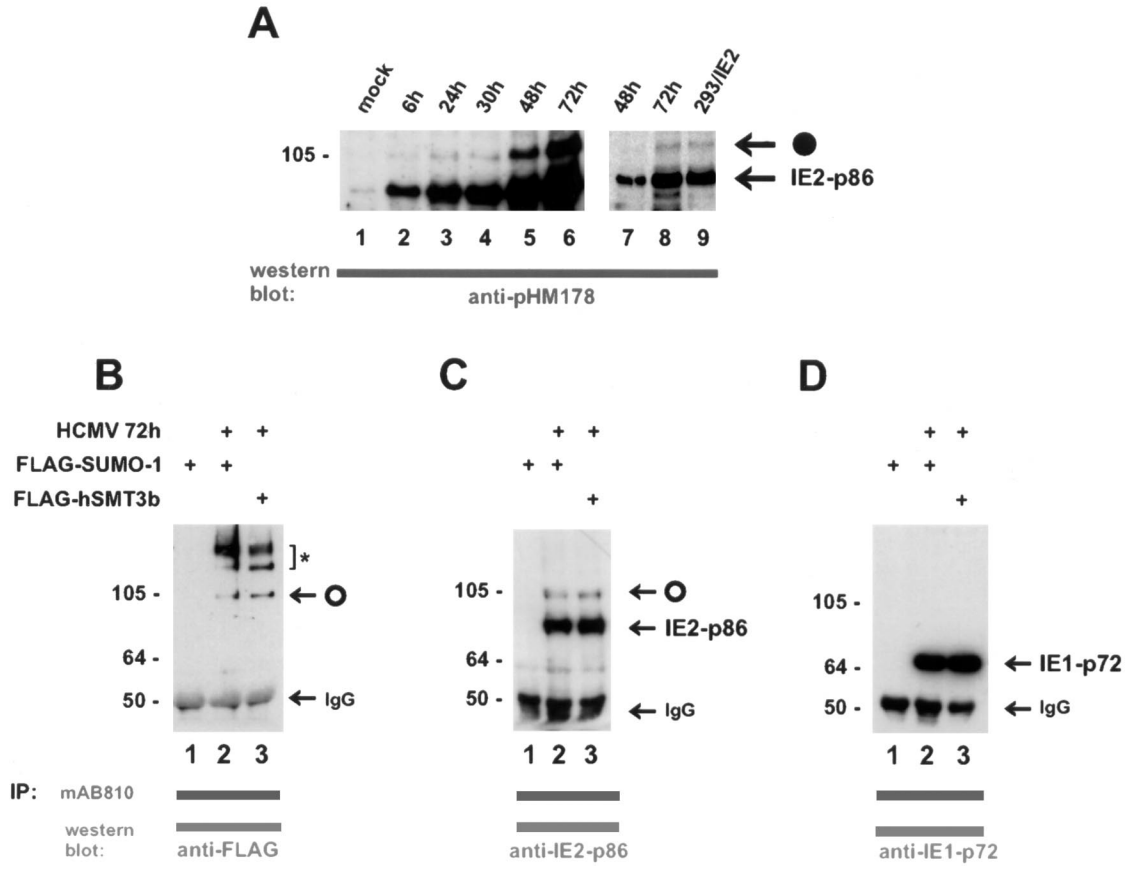


FIG. 5. Evidence for a covalent modification of HCMV IE2-p86 in infected HFFs. (A) Western blot analysis using lysates from infected HFFs and transfected 293 cells. HFFs were either mock infected or infected with the laboratory strain AD169 for the indicated time periods. Cells were lysed in SDS sample buffer, and the lysates were fractionated by SDS-PAGE (10% gel) followed by Western blot analysis using the anti-pHM178 polyclonal antiserum directed against exon 5 of IE2-p86. Lanes: 1, lysate from mock-infected HFFs; 2 to 6, lysates from HFFs that were infected with HCMV for 6, 24, 30, 48, and 72 h, respectively; 7 to 8; lysates from HFFs that were infected with HCMV for 48 and 72 h, respectively; 9, lysate from 293 cells that were transfected with the IE2-p86 expression vector pHM134. The 105-kDa isoform of IE2 is indicated by a closed circle. (B to D) Immunoprecipitation of IE1 or IE2 conjugates from HCMV-infected HFFs. HFFs were transfected with an expression vector encoding FLAG-SUMO-1 or FLAG-hSMT3b. The day after transfection, cells were either mock infected or infected with HCMV strain AD169 for 72 h as indicated. Thereafter, immunoprecipitation was performed with MAb 810, recognizing an epitope common to both IE2-p86 and IE1-p72. Western blot analysis was performed using the anti-FLAG MAb in order to detect SUMO-1 or hSMT3b (B), the IE2-p86 polyclonal antiserum in order to detect IE2 proteins (C), and MAb p63-27 in order to detect IE1 proteins (D). Transfection of HFFs was performed with plasmids encoding either FLAG-SUMO-1 (lanes 1 and 2) or FLAG-hSMT3b (lane 3). The modification of IE2-p86 by the FLAG-UbH proteins is indicated by open circles.

IE2(290-548), IE2(310-548), and IE2(329-548) were cloned into the pSuperCATCH-NLS vector in order to ensure a nuclear localization of the encoded proteins. This was done since a recent study suggested that a nuclear localization of a given target protein is necessary for SUMOylation (62). As determined by indirect immunofluorescence analysis, all constructs exhibited a strictly nuclear localization (data not shown). Then, each of the constructs was cotransfected with FLAG-SUMO-1 or FLAG-hSMT3b in 293 cells followed by Western blot analysis with the IE2-p86 antiserum in order to examine whether the mutants were still conjugated (data not shown). Thus, we were able to map the conjugation domain in IE2 between amino acid 173 and 290 (Fig. 6A).

Peptide sequence similarity analysis within this domain showed a striking homology between amino acids 169 to 182 of IE2-p86 and the already published SUMO-1 conjugation site in RanGAP1 (42). In addition, this sequence also exhibits similarities to the respective SUMOylation sites within IκBα, Sp100, and PML (Fig. 6B) (13, 33, 62). Sternsdorf et al. (62) proposed the consensus sequence (I/L)KxE for SUMOylation in which the lysine residue is linked to the ubiquitin-like moiety (62). Interestingly, two (I/L)KxE motifs could be found in IE2-p86 within amino acids 169 to 182, in which both lysine 175 and lysine 180 might serve as acceptors for conjugation independently. To further investigate this amino acid sequence, we performed a PCR mutagenesis by which we replaced either lysine 175 (IE2mut175) or lysine 185 (IE2mut180) separately or both simultaneously (IE2mut175+180) by arginine. The replacement of lysine by arginine was chosen in order to maintain a basic charged residue at each amino acid position. Additionally, we created an IE2 mutant with an internal deletion of amino acid residues 174 to 181 (IE2del174-181) (Fig. 7A). All mutants were transfected into 293 cells in the absence or presence of FLAG-SUMO-1, and cell lysates were analyzed by Western blotting using the IE2-p86 antiserum (Fig. 7B). As observed for wild-type IE2, each of the lysine single point mutants was still modified by an endogenous moiety in the absence of FLAG-SUMO-1 (Fig. 7B, lanes 2, 4, and 6) and could also be conjugated with FLAG-SUMO-1, which again resulted in a doublet at 105 kDa (lanes 3, 5, and 7). In contrast, neither the internal deletion mutant IE2del174-181 nor the

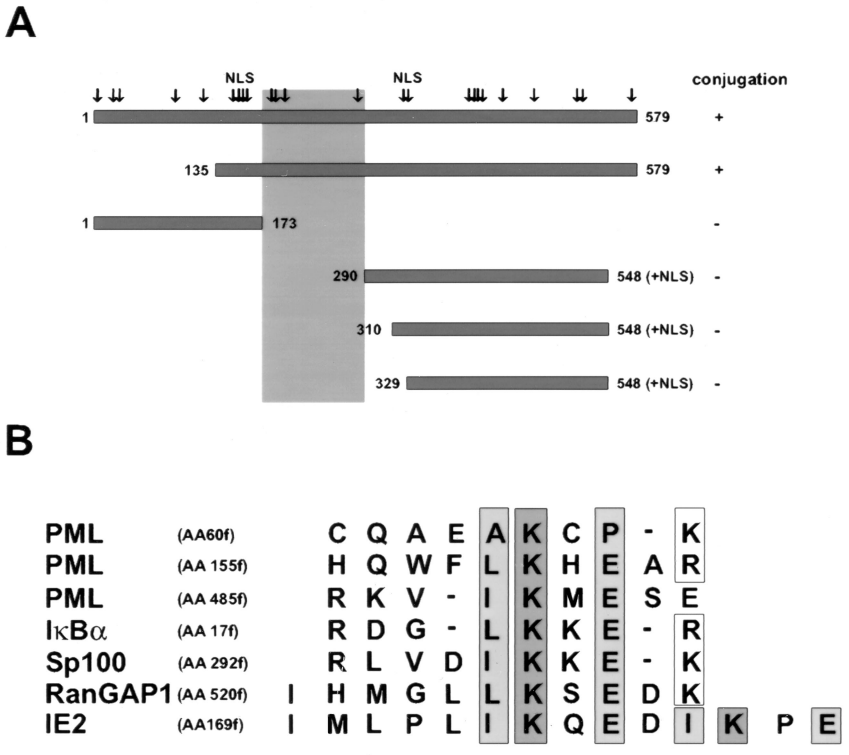


FIG. 6. Mapping of lysine residues responsible for SUMOylation of HCMV IE2-p86. (A) Schematic overview depicting the IE2 deletion mutants used. Lysine residues in IE2-p86 are indicated by arrows; the positions of nuclear localization signals (NLS) within IE2 are also indicated. The eucaryotic expression vectors were transfected into 293 cells either alone or in combination with FLAG-SUMO-1 and analyzed for conjugation by Western blotting, indicated by + (conjugation detectable) or − (no conjugation observed). The IE2 mutants comprising amino acids 290 to 548, 310 to 548, and 329 to 548 were cloned into the pSuperCATCH-NLS vector to ensure nuclear localization. (B) Sequence similarity analysis between already determined SUMOylation sites in cellular proteins and HCMV IE2-p86. The proposed SUMOylation consensus motif is indicated by boxes; the lysine residues responsible for isopeptide bond formation are shaded darker.

double amino acid point mutant IE2mut175+180 was modified (lanes 8 to 11).

To further confirm these results, we performed immunoprecipitations after cotransfection of the individual mutants in combination with FLAG-SUMO-1 or FLAG-hSMT3b. Consistent with the observations of Western blot experiments, the single amino acid mutants IE2mut175 and IE2mut180 were still conjugated with FLAG-SUMO-1 or FLAG-hSMT3b (Fig. 8A, lanes 7, 8, 11, and 12), whereas the internal deletion mutant as well as the double amino acid mutant IE2mut175+180 were not modified by either of the two UbH proteins (lanes 14, 15, 18, and 19). In contrast, all IE2 mutants were still able to interact with FLAG-UL84 under native lysis conditions (lanes 5, 9, 13, and 17), indicating that the conformation of the IE2-protein was not severely affected by the introduced mutations. This was further strengthened by coimmunoprecipitation experiments after transfection of the IE2 mutants in combination with a FLAG-tagged, N-terminally truncated IE2 mutant lacking the epitope as recognized by the IE2 MAb 810. After precipitation with MAb 810, this FLAG-tagged IE2 could be detected in Western blot analysis using the anti-FLAG antibody, indicating that all mutants were still able to dimerize (Fig. 8B).

These experiments demonstrate that the SUMO-1/hSMT3b conjugation site within IE2 is located between amino acids 174 and 181. Within this sequence, two separate modification sites (K175 and K180) could be identified.

SUMOylation is not necessary for colocalization of IE2 with ND10. As published previously, both IE1 and IE2 proteins of HCMV are targeted to ND10 domains within a few hours after infection (1, 36, 71). For PML, the first identified ND10-associated protein, it was observed that SUMOylation is required for its accumulation in these subnuclear domains (49). Having generated an IE2 mutant which is no longer conjugated by SUMO-1 and hSMT3b, we addressed the question of whether SUMOylation affects the targeting of IE2-p86 to ND10 structures. For this purpose, wild-type IE2-p86 fused to GFP (IE2-GFP) and the IE2 mutants described before were transfected into HFFs. Immunofluorescence analysis was carried out using the Sp26 antiserum recognizing Sp100 in order to detect endogenous ND10 structures and MAb 810 to detect the IE2 mutants. Wild-type IE2 was visible through its GFP moiety. As evident from Fig. 9, wild-type IE2-p86 and the single amino acid mutants exhibited a diffuse nuclear staining with some brighter speckles correlating with Sp100 localization (Fig. 9A to F). Transfection of the double amino acid mutant that is defective for SUMO-1 or hSMT3b conjugation resulted in a predominantly speckled nuclear staining that showed a perfect colocalization with Sp100 nuclear dots. Thus, modification by either SUMO-1 or hSMT3b is not a determinant for accumulation of IE2-p86 within ND10 structures.

Influence of the SUMO-1/hSMT3b modification on IE2-mediated transactivation. Since IE2-p86 is known as a strong transactivator of viral as well as cellular promoters, we addressed the question of whether the IE2 mutants described above showed any difference in transcriptional activity compared with wild-type IE2-p86. For this purpose, permissive U373MG cells were transiently transfected with reporter plas-

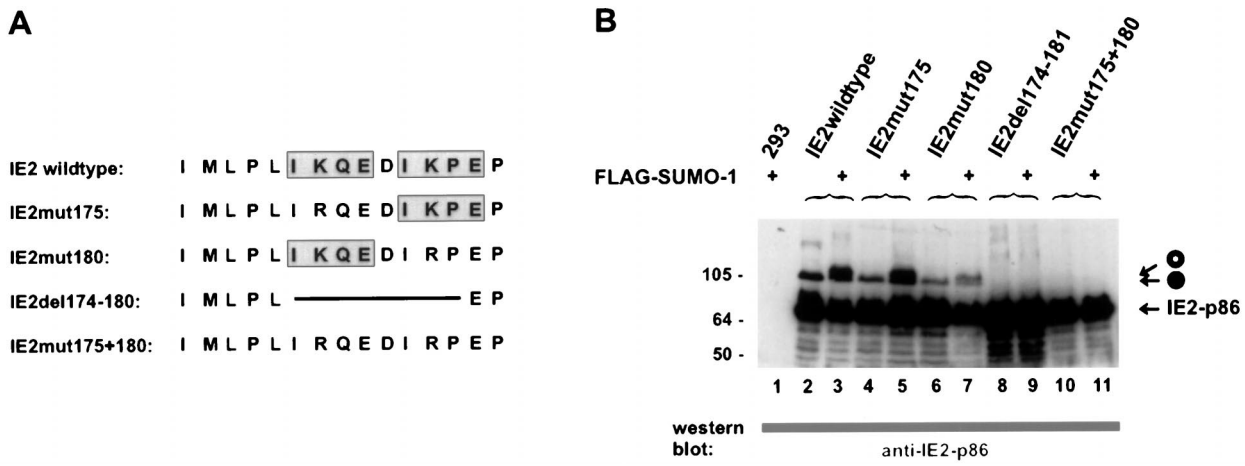


FIG. 7. Interaction of IE2-p86 amino acid mutants with UbH proteins. (A) Schematic overview of the amino acid deletions or substitutions in IE2-p86 generated by PCR mutagenesis. Potential SUMOylation consensus motifs are indicated by boxes. (B) Western blot analysis of the individual IE2-p86 mutants. 293 cells were transfected with expression vectors encoding either wild-type IE2-p86 or the individual mutants alone or in combination with a vector encoding FLAG-SUMO-1 as indicated. Cell lysates were analyzed by immunoblotting using the IE2-p86 polyclonal antiserum. Lanes: 1, transfection with a vector encoding FLAG-SUMO-1 alone; 2, transfection with IE2-p86 expression vector pHM134; 3, transfection with pHM134 and pFLAG-SUMO-1; 4, transfection with a vector encoding IE2mut175 alone; 5, transfection with pIE2mut175 and pFLAG-SUMO-1; 6, transfection with a vector encoding IE2mut180 alone; 7, transfection with pIE2mut180 and pFLAG-SUMO-1; 8, transfection with a vector encoding IE2del174-181 alone; 9, transfection with pIE2del174-181 and pFLAG-SUMO-1; 10, transfection with a vector encoding IE2mut175+180 alone; 11, transfection with pIE2mut175+180 and pFLAG-SUMO-1. The IE2-p86 modification by an endogenous UbH moiety is indicated by a closed circle; the modification by cotransfected FLAG-SUMO-1 is depicted by an open circle.

mids containing the luciferase gene under the control of two IE2-responsive early promoters of HCMV. The first reporter construct, termed pHM142, contained the UL112/113 promoter which had previously been described as a highly IE2-responsive element (5, 56). The second plasmid, pHM571, contained the promoter driving expression of the UL84 gene of HCMV, which is also strongly transactivated by IE2-p86 (S. Flöss and T. Stamminger, unpublished data). After cotransfection of these reporter plasmids with either the wild-type IE2-p86 expression vector pHM134 or the IE2 mutants IE2mut175, IE2mut180, and IE2mut175+180, luciferase activity was determined in cell extracts. Each experiment was performed in triplicate and repeated at least three times. Transcriptional activation was determined by calculating fold activation based on the activity of the reporter plasmids after cotransfection with the expression vector pCB6 containing the HCMV IE1/2 enhancer-promoter without a coding sequence. As shown in Fig. 10, both reporter constructs were activated by wild-type IE2 approximately 230- to 300-fold. There was no significant difference of activation mediated by IE2mut175 and IE2mut180 compared to wild-type IE2. In contrast, a strong decrease in activation mediated by IE2mut175+180 was observed for both promoters that were tested. Sommer et al. (56) had shown in a previous study using the UL112/113 promoter in U373MG cells that an internal deletion of amino acids 135 to 290 (in mutant IE86ΔSX) within IE2-p86 reduced the transactivation potential about 50% (56). We therefore included this mutant, kindly provided by D. Spector (San Diego, Calif.) in our luciferase experiments. As is evident from Fig. 10, there is no significant difference in stimulation of the UL112/113-promoter mediated by IE2mut175+180 compared to the IE86ΔSX mutant or the internal deletion mutant IE2del174-181. A similar reduction in transactivation was observed when we used the HIV LTR as a heterologous promoter in combination with the SUMOylation-defective mutants of IE2-p86 (Fig. 10C). Western blot experiments excluded that a reduced expression level of the mutant proteins was responsible for their loss of transactivation capacity (Fig. 10D). In summary, these results suggest that a covalent modification of IE2-p86 by UbH proteins is critical for the transactivation capacity of IE2-p86.

DISCUSSION

The IE2-p86 protein of HCMV is a multifunctional regulator of viral as well as cellular gene expression. Protein-protein interactions are thought to play a major role in IE2-mediated regulation of this broad spectrum of promoters, and it has been reported that IE2-p86 is able to interact with at least 15 cellular factors (18), some of which have been identified. Known interaction partners include members of the basal transcription machinery such as TATA-binding protein and TFIIB (10, 25), several transcription factors (e.g., AP-1, Egr-1, and CREB) (37, 55, 74), and the cell cycle regulators pRb and p53 (24, 57). However, since the majority of those binding partners are still unknown, we performed a yeast two-hybrid screen using the carboxy terminus of IE2 as a bait. An N-terminally truncated protein was chosen since this IE2 version lacking the N-terminal acidic transcriptional activation domain did not activate the reporter genes in yeast when fused to the GAL4 DNA-binding domain. In addition, published data indicate that the C terminus of IE2 as used in our yeast two-hybrid screen contains important protein interaction motifs (10, 24, 56). Furthermore, we were able to observe an interaction of this bait construct after coexpression with the viral UL84 protein fused to the GAL4 activation domain as well as a dimerization with an IE2-GAL4 activation domain fusion protein. This suggests a native conformation of the bait protein in fusion with the GAL4 DNA-binding domain which allows for an interaction with other proteins.

Using the yeast two-hybrid screen, we were able to isolate several copies of two polypeptides termed SUMO-1 and hSMT3b as well as the hUBC9 enzyme as specific interaction partners of IE2 in yeast. The proteins SUMO-1 and hSMT3b are highly homologous, being 46% identical on the amino acid level. Since they exhibit a low but significant homology to

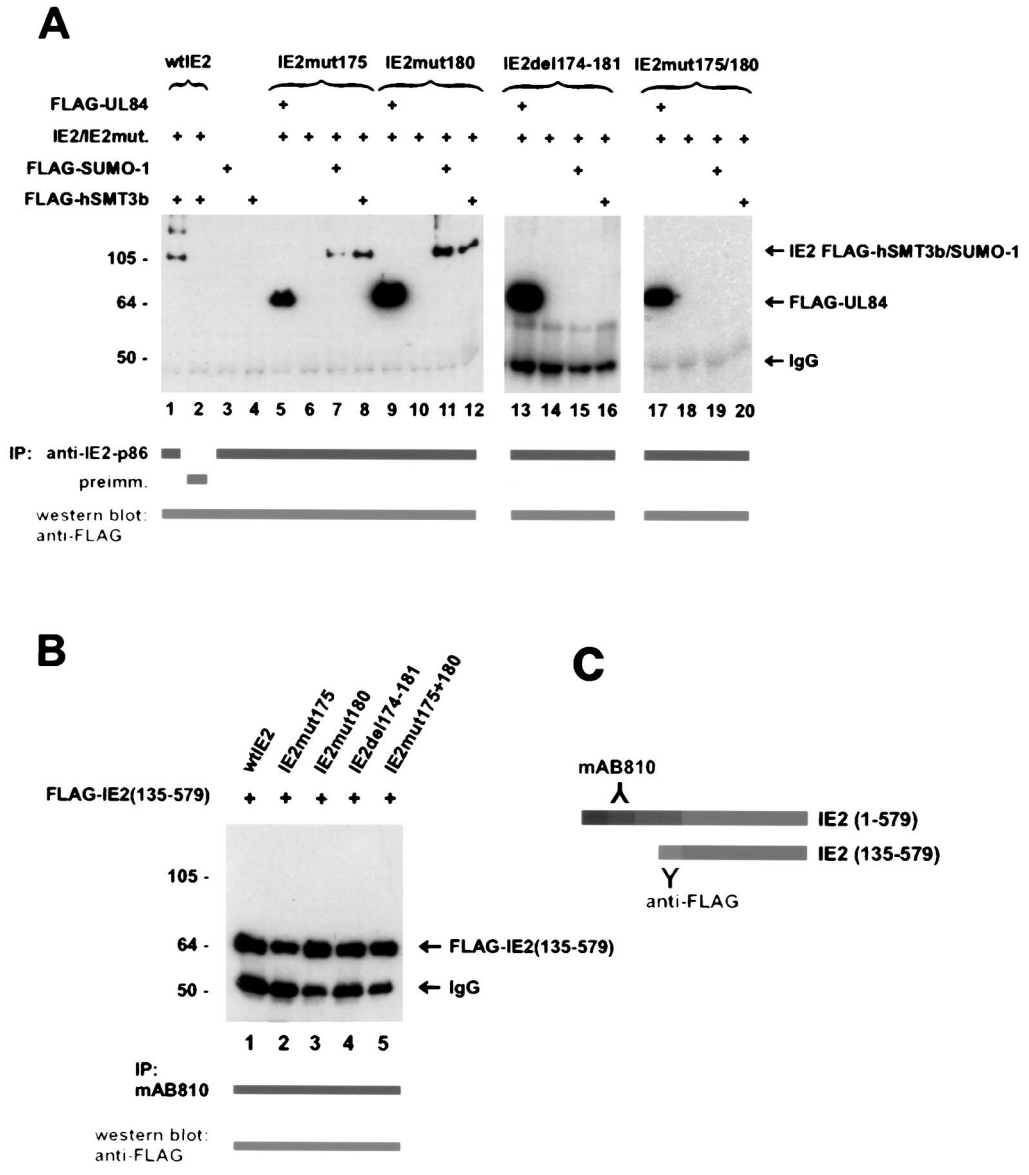


FIG. 8. Analysis of IE2-p86 mutants by immunoprecipitation experiments. 293 cells were transfected with the indicated expression vectors, and immunoprecipitations were performed using the IE2-p86 polyclonal antiserum or preimmune serum (A) or MAb 810 (panel B). (A) Conjugation of IE2-p86 mutants with UbH proteins. Coprecipitated interactor proteins were detected in Western blot experiments using the anti-FLAG MAb M2. For lanes 5, 9, 13, and 17, cell lysis was performed with NP-40 lysis buffer; for all other lanes, SDS-containing buffer was used (see Materials and Methods). (B) Evidence for dimerization capability of the individual IE2-p86 mutants. 293 cells were transfected with a vector encoding an N-terminally truncated IE2-protein [FLAG-IE2(135-579)] in combination with expression vectors for either IE2-p86 wild-type protein (wtIE2) or a mutated variant of IE2 (IE2mut175, IE2mut180, IE2del174-181, or IE2mut175+180). Cells were lysed in NP-40 lysis buffer, and immunoprecipitations were performed with MAb 810, which recognizes an N-terminal epitope within IE2-p86 or the individual mutants that are not contained in the FLAG-IE2(135-579) protein (see panel C). Thereafter, Western blot analysis was performed using the anti-FLAG MAb in order to specifically detect the FLAG-IE2(135-579) protein.

ubiquitin, they are referred to as UbH proteins (7, 44, 45). Similar to ubiquitin, these two UbH polypeptides can be covalently attached to a variety of mostly nuclear target proteins by an enzymatic process with analogy to protein ubiquitinylation (32, 34). This covalent linkage involves the hUBC9 enzyme, a member of the E2 ubiquitin-conjugating enzyme family with a strict specificity for the substrates SUMO-1 and hSMT3b (54). Hereby, hUBC9 mediates the formation of an isopeptide bond between the carboxy terminus of the respective UbH and the amino group of a lysine residue in the target protein (42). However, in contrast to ubiquitinylation, there is currently no evidence that the proteins modified by SUMO-1 or hSMT3b are targeted for destruction via the proteasome (70).

Having identified UbH polypeptides and hUBC9 as specific interaction partners of IE2, we wondered whether IE2-p86 could be modified by covalent linkage to SUMO-1 or hSMT3b. By Western blot experiments using lysates derived from transfected 293 cells, we were able to demonstrate a modification of IE2-p86 after coexpression with a FLAG-tagged SUMO-1 or hSMT3b resulting in a 105-kDa IE2 isoform, which could be detected using two different IE2-specific antibodies. To reliably detect this isoform, it turned out to be critical to lyse cells under strictly denaturing conditions, since SUMO-1 or

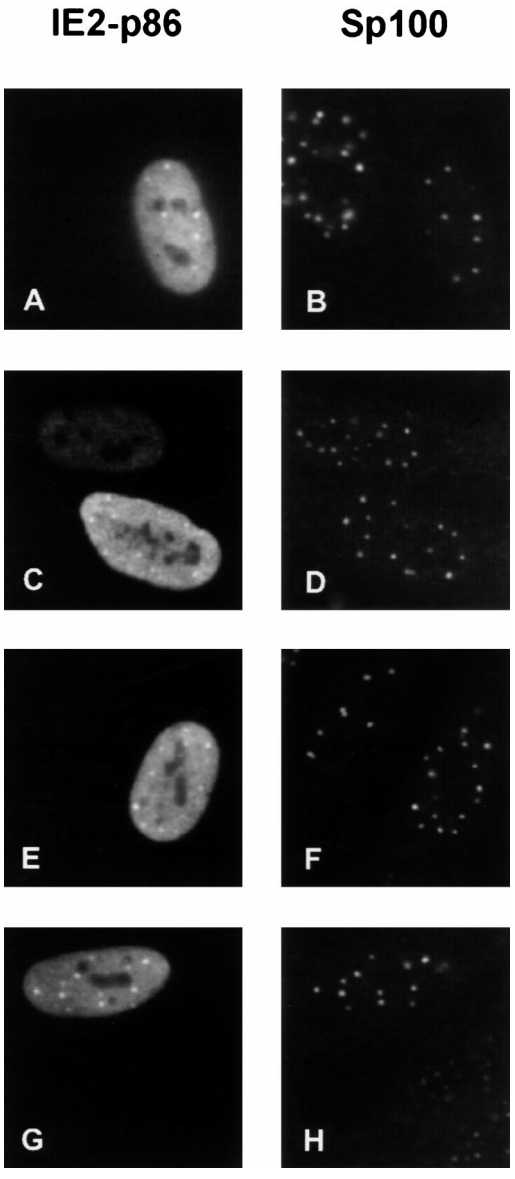


FIG. 9. Subcellular localization of IE2-p86 and mutant IE2 proteins in HFFs. HFFs grown on coverslips were transfected with expression vectors encoding the wild-type IE2-p86 protein fused to GFP (A and B) or IE2 mutant IE2mut175 (C and D), IE2mut180 (E and F), or IE2mut175+180 (G and H). Indirect immunofluorescence was performed with MAb 810 and an FITC-conjugated anti-mouse secondary antibody in order to detect the IE2 mutants (C, E, and G). Wild-type IE2 was detected through its GFP moiety (A). Additionally, costaining was performed with a polyclonal Sp26 antiserum directed against Sp100 and a TRITC-conjugated anti-rabbit conjugated secondary antibody in order to detect ND10 (B, D, F, and H).

hSMT3b conjugates are highly unstable in NP-40 lysis buffer most probably due to a deSUMOylation activity in cell lysates (13). Under denaturing lysis conditions, a 105-kDa IE2 isoform could even be detected after expression of IE2-p86 in the absence of a FLAG-tagged SUMO-1, most probably due to a modification by an endogenous UbH moiety.

Furthermore, we were able to immunoprecipitate the conjugated 105-kDa IE2 isoform from lysates of transfected 293 cells under denaturing conditions, thus further confirming the covalent nature of the interaction. Interestingly, two additional FLAG-reactive bands of 130 and 150 kDa could reproducibly be observed after immunoprecipitation with IE2-specific antibodies. Since these signals were detectable only in reactions containing both IE2-p86 and the respective FLAG-UbH polypeptide, it is highly unlikely that they are the result of nonspecific binding of FLAG-tagged proteins to primary antibodies or to the Sepharose beads. Surprisingly, these bands were not detected in Western blot analyses using various IE2-specific antibodies, suggesting that they do not represent additional IE2 isoforms. A similar observation of a higher-molecular-weight protein species has also been described for IκBα/SUMO-1 conjugates (13). Whether these signals are due to cellular UbH-conjugated proteins that strongly interact with IE2-p86 is not known.

After having demonstrated that IE2-p86 can be covalently modified by SUMO-1 or hSMT3b after transient expression of each protein, we wanted to know whether this conjugation could also occur in HCMV-infected human fibroblasts. By Western blot analysis, we were able to show that a 105-kDa IE2 isoform is detectable during the whole time course of the HCMV replicative cycle, with the most prominent signal occurring at late times after infection. Moreover, we could immunoprecipitate the 105-kDa IE2 isoform after HCMV infection of fibroblasts that had been transfected with expression vectors for either the FLAG-tagged SUMO-1 or hSMT3b, demonstrating the stability of these isoforms under conditions of viral infection.

In summary, these experiments show that IE2-p86 can be covalently coupled to SUMO-1 or hSMT3b both after cotransfection and during viral infection. At present, we are not able to definitely demonstrate whether the endogenous moiety that is coupled to IE2-p86 corresponds to SUMO-1 or hSMT3b. This is due to several reasons. First, since no specific antibody against hSMT3b is available as yet, it is impossible to detect the endogenous protein. Second, the only available antibody against SUMO-1, MAb 21C7 (45), does not react with SUMO-1-conjugated IE2 even in experiments where IE2-p86 was cotransfected with FLAG-tagged SUMO-1, which allowed for a reliable monitoring of the conjugation by using an anti-FLAG MAb (data not shown). This may be due to the fact that this antibody has been generated against RanGAP1-conjugated SUMO-1 and may require an epitope shared by SUMO-1 and RanGAP1 for high-affinity binding, thus making the detection of other SUMO-1-conjugated proteins difficult. Alternatively, the epitope recognized by MAb 21C7 may not be accessible in the IE2-p86-SUMO-1 conjugate. Therefore, the final evidence for the nature of the endogenous UbH moiety conjugated to IE2-p86 awaits the availability of novel reagents allowing the detection of distinct species of UbH polypeptides.

IE2-p86 is not the only protein encoded by HCMV that can be modified by covalent coupling to SUMO-1 or hSMT3b. Consistent with a recent publication by Müller and Dejean (48), we observed a higher-molecular-weight species of the IE1-p72 transactivator after cotransfection of a vector encoding FLAG-tagged SUMO-1. This does not reflect a general, nonspecific modification after overexpression of UbH molecules since several other viral proteins (e.g., pUL84, pUL69, and pUL26) were not coupled under those conditions. However, we noticed differences in the modification of IE1-p72 in comparison to IE2-p86. First, no endogenously modified protein species could be detected for IE1-p72 after transfection of an IE1 expression vector alone. Second, we were not able to immunoprecipitate an IE1-p72/SUMO-1 conjugate after HCMV infection of cells that had been transfected with a vector for FLAG-tagged SUMO-1/hSMT3b. At present, we do not know whether these differences are due to a higher instability of the respective IE1 conjugate or reflect a differential

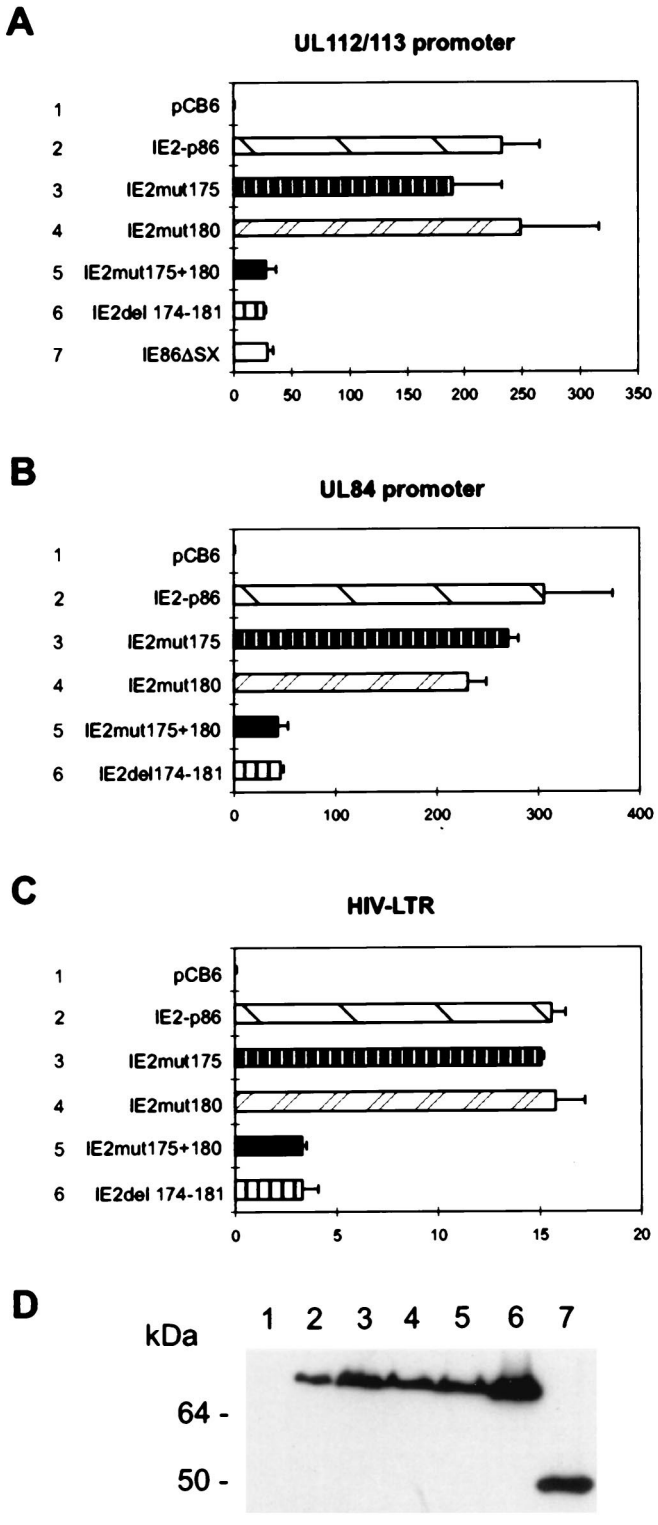


FIG. 10. Luciferase analysis after cotransfection of luciferase reporter constructs carrying viral early promoters or the HIV-1 LTR with expression plasmids for various IE2 mutants. U373MG cells were transfected with luciferase reporter constructs carrying the viral early promoters of the UL112/113 gene region (A, lanes 1 to 7), the UL84 gene (B, lanes 1 to 6), or the HIV LTR (C, lanes 1 to 6). Lanes: 1, cotransfection was performed with the empty expression vector pCB6; 2, cotransfection was performed with expression vector pHM134 encoding wild-type IE2-p86; 3, cotransfection was performed with the expression vector for IE2 mutant IE2mut175; 4, cotransfection was performed with the expression vector for IE2 mutant IE2mut180; 5, cotransfection was performed with the expression vector for IE2 mutant IE2mut175+180; 6, cotransfection was performed with the internal deletion mutant IE2del174-181; 7, cotransfection was performed with the internal deletion mutant IE86ΔSX (kindly provided by D. Spector) (56). Each experiment was performed in triplicate and was repeated at least three times. Fold activation was calculated relative to the basal activity of each reporter construct after cotransfection with the empty pCB6 vector. (D) Western blot analysis of 293 cell extracts after transfection of various IE2 expression plasmids using the IE2-specific MAb 810. Lanes: 1, transfection was performed with expression vector pCB6; 2, transfection was performed with vector pHM134; 3, transfection was performed with the vector encoding IE2mut175; 4, transfection was performed with the vector encoding IE2mut180; 5, transfection was performed with the vector encoding IE2mut175+180; 6, transfection was performed with the internal deletion mutant IE2del174-181; 7, transfection was performed with the internal deletion mutant IE86ΔSX.

regulation of SUMOylation during the HCMV replicative cycle.

To identify the lysine residue within IE2-p86 which serves as an acceptor for covalent coupling by UbH moieties, we used a panel of N- and C-terminal IE2 deletion mutants. Hereby, we were able to localize a domain within IE2-p86 that shares a high similarity to already published SUMOylation sites of other cellular proteins (13, 33, 42, 62). By PCR mutagenesis we could show that this domain contains two SUMOylation motifs involving amino acids 175 and 180; only a double mutation of both lysine residues abolished modification by SUMO-1 or hSMT3b. The loss of the endogenous 105 kDa IE2-isoform after mutation of both lysine residues can serve as an additional strong argument that a UbH-like protein is responsible for this modification. As we observed that mutation of either conjugation site alone still results in a 105-kDa IE2 isoform, we believe that both lysine residues can serve as acceptors for modification. However, we never detected a 130-kDa isoform in Western blot analysis which would be consistent with a simultaneous linkage of two UbH moieties to IE2-p86. Therefore, we favor the hypothesis that these sites are used alternatively, probably because of steric hindrance: once a modification has taken place, the second site would no longer be accessible for coupling. However, it remains to be determined why IE2-p86 possesses these two motifs in an immediate vicinity.

Finally, we were interested in the function of IE2 SUMOylation. For RanGAP1, a protein involved in nuclear transport, it was shown that only the SUMO-1-modified isoform p90 is localized at the nuclear pore complex whereas the nonmodified form p70 has a cytoplasmic distribution (41, 45). As HCMV IE2-p86 is strictly nuclear and as the conjugation sites are not contained within the nuclear localization signals of IE2 (73), a targeting to the nuclear pore complex as a consequence of modification is probably not the function of IE2 SUMOylation. For the inhibitor of NFκB, IκBα, it was shown that covalent attachment of SUMO-1 protected against signal-induced degradation (13). However, since protein steady-state levels of the SUMOylation-defective IE2 double mutant did not differ significantly from those of the single amino acid mutants or the wild-type protein, a major influence of SUMOylation on the half-life of IE2 appears to be unlikely. Recently, two proteins of ND10 (also termed PML oncogenic domain or Kr bodies), PML and Sp100, have turned out to be SUMO-1 conjugated (33, 49, 63). For PML, the first identified ND10-associated protein, it was observed that SUMOylation is necessary for its accumulation within these domains (49). In contrast, the Sp100 protein is present in ND10 even when SUMOylation is abolished, indicating that covalent coupling with SUMO-1 does not necessarily refer to a localization within nuclear bodies (62). Having generated an IE2 mutant

which is no longer conjugated, we showed that SUMOylation of IE2-p86 is not required for its accumulation within ND10. This suggests that SUMOylation is not a common pathway for protein targeting to ND10 domains.

Apart from the known cellular target proteins for SUMOylation as mentioned above, several publications have described an interaction of the hUBC9 enzyme with various bait proteins in yeast two-hybrid screens (27, 28, 66, 72). Since the only function of hUBC9 known so far is the attachment of UbH moieties to target factors, one might speculate that at least some of those bait proteins are likewise SUMOylated. Interestingly, several of those bait proteins, e.g., Ets-1 (27), WT-1 (66), and viral regulatory proteins like the adenovirus E1a protein (28), exhibit transcriptional activity. For some of these factors it was reported that cotransfection of a hUBC9 expression vector resulted in increased transactivation of a given reporter construct (27). This implicates a potential role of modification by UbH polypeptides for transcriptional regulation and raised the hypothesis that SUMOylation might be of a more general importance for transcription factors. Consistent with this, two recent publications demonstrated that a modification of p53 by SUMO-1 resulted in enhanced transactivation by p53 (22, 53). Therefore, we investigated whether the IE2 mutants described above showed differences in transactivation potential from wild-type IE2-p86. Hereby, we observed a drastic reduction of transactivation mediated by the double amino acid mutant which is negative for SUMOylation, whereas the single amino acid mutants that are still coupled to UbH proteins were not defective. This suggests that SUMOylation is of importance for IE2-mediated transactivation. At present, we cannot totally exclude that the simultaneous mutation of two lysine residues within this region results in an alteration of IE2 protein conformation leading to a reduction in transactivation levels, although each of the single amino acid mutants was fully functional. However, we performed several different approaches to confirm the integrity of the double mutant protein structure. First, we substituted each lysine residue against another basic amino acid, arginine, in order to maintain the basic charge within the mutated sequence in IE2-p86. Second, we demonstrated that all IE2 mutants could still interact with the viral pUL84 protein, as confirmed by coimmunoprecipitation analysis. Previous experiments in our laboratory suggested that an extended domain within IE2-p86 is required for binding to pUL84 (T. Stamminger, unpublished observation), indicating that the structure of the individual mutants still allows for an interaction. Third, the individual mutants are still able to dimerize, as shown by coimmunoprecipitation experiments. Last, we were able to confirm the interaction between the individual mutants and the hUBC9 enzyme in yeast (H. Hofmann and T. Stamminger, unpublished data). This is of particular importance since it proves that mutation of lysine residues which abolishes SUMOylation does not interfere with hUBC9 interaction, which in turn would prevent modification.

Extensive investigations by other laboratories have characterized domains within IE2-p86 that are necessary for transactivation. Hereby, it was shown that both an amino-terminal domain comprising amino acids 1 to 98 as well as the carboxy-terminal half of the IE2-p86 protein are required for stimulation of early HCMV promoters (43, 50, 56, 60). Additionally, Yeung et al. reported that amino acids 169 to 194 are necessary for IE2-p86 mediated transactivation of the HIV LTR (73). Consistent with this, our data show that amino acids 175 and 180 are important for stimulation of the early HCMV promoters of the UL112/113 and UL84 gene regions as well as for stimulation of the HIV LTR. The UL112/113 promoter had also been the subject of studies by Sommer et al. who showed that an internal deletion of amino acids 135 to 290 within IE2-p86 (in deletion mutant IE86ΔSX) resulted in an approximately 50% reduction in the transactivation potential compared to wild-type IE2-p86 (56). Luciferase experiments using a reporter construct under control of the UL112/113 promoter in combination with either the IE86ΔSX mutant or the internal deletion mutant IE2del174-181 showed a transactivation potential similar to that observed with the SUMOylation negative-double mutant IE2mut175+180.

Our current model concerning the function of IE2 SUMOylation includes the UbH residue as an additional protein interaction motif for other cellular cofactors, which in turn might also be modified by UbHs (7). To back up this hypothesis, it would be necessary to identify cellular cofactors which preferentially interact with wild-type IE2 but not with the double amino acid mutant. To determine the importance of IE2 SUMOylation for the lytic replicative cycle of HCMV, the construction of a recombinant virus carrying the double amino acid mutation in the IE2 open reading frame is in progress.

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